Chronic Administration of Amiodarone Does Not Affect $\text{Na}^+/\text{Ca}^{2+}$ Exchange Current in Guinea Pig Cardiac Ventricular Myocytes

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ABSTRACT—We investigated chronic effects of amiodarone on $\text{Na}^+/\text{Ca}^{2+}$ exchange current (I_{NCX}) and on the level of $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX1) mRNA in guinea pig ventricular myocytes using the whole-cell clamp technique and RT-PCR analysis, respectively. Guinea pigs were intraperitoneally injected with 80 mg/kg per day of amiodarone or the vehicle (saline) for 1 or 4 weeks. Single ventricular cells were isolated from the hearts of both groups of animals. Action potential duration at 90% repolarization level was prolonged to 143% and 165% of the control values by treatment with amiodarone for 1 and 4 weeks, respectively. I_{NCX} density and the level of NCX1 mRNA were not significantly changed by chronic treatment with amiodarone. The level of thyroid hormone (T_4) within the blood was not changed by the treatments. These results suggest that chronic treatment with amiodarone does not affect the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, with respect to the level of its mRNA and current density in guinea pig ventricular myocytes.

Keywords: Amiodarone, Cardiac myocyte (guinea pig), Chronic effect, $\text{Na}^+/\text{Ca}^{2+}$ exchange current

Amiodarone was first approved by the United States Food and Drug Administration in 1985 for the treatment of serious ventricular arrhythmia (1). It was originally classified as a class III antiarrhythmic drug in Vaughan Williams classification, but it also has multiple actions including Na^+ -, K^+ - and Ca^{2+} -channel blocking effects (2). Amiodarone also reduces β -adrenergic receptors (2).

The acute and long-term effects of amiodarone have been shown to differ significantly in cardiac electrophysiological effects (3, 4). The most striking difference is that chronic treatment prolongs the action potential duration (APD) in ventricular cells or QT intervals in electrocardiogram (5–10), while acute treatment shortens APD in ventricular cells (11, 12) and does not change APD in atrial cells (13).

Chronic treatment with amiodarone inhibits various K^+ currents such as the 4-AP-sensitive transient outward K^+ current (I_{to}), the delayed rectifier K^+ currents (I_{K} , I_{Kr} , I_{Ks}) and the inwardly rectifying K^+ current (I_{K1}) (12, 14–16). Chronic treatment also causes a down-regulation of $\text{Kv}1.5$ K^+ channel mRNA synthesis in rat heart (17). L-type Ca^{2+}

current (12) and the Na^+/K^+ pump (18, 19) are also inhibited. Another unique feature of amiodarone is that it contains iodine and has a structural similarity to thyroid hormone. Thus, chronic amiodarone treatment could cause hyper- or hypothyroidism (20).

$\text{Na}^+/\text{Ca}^{2+}$ exchange is a major mechanism for extruding Ca^{2+} from cardiac myocytes (21–24). We recently reported that acute administration of amiodarone in a therapeutic concentration range inhibited $\text{Na}^+/\text{Ca}^{2+}$ exchange current (I_{NCX}) in single guinea pig cardiac ventricular myocytes (25). The inhibitory effect of amiodarone was not attenuated by intracellular trypsin treatment, which is similar to that of bepridil (26) and 2,3-butane-dione monoxime (BDM) (27). However, the effects of chronic administration of amiodarone on I_{NCX} have not been thoroughly investigated. In the present study, we examined the effect of chronic treatment with amiodarone for 1 or 4 weeks on I_{NCX} with the whole-cell voltage clamp technique and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX1) mRNA with the RT-PCR method in guinea pig cardiac ventricular cells.

MATERIALS AND METHODS

Animals and amiodarone treatment

All experiments were performed in accordance with the regulations of the Animal Research Committee of the

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School of Medicine, Fukushima Medical University. Male guinea pigs weighing 250–300 g were used. Guinea pigs in the amiodarone treatment group were injected intraperitoneally with 80 mg/kg per day of amiodarone every day for 1 or 4 weeks, and those in the saline group, with saline daily for 1 or 4 weeks. The average body weight of animals in the saline group was 354 ± 17 g ($n = 6$) after 1 week and 483 ± 44 g ($n = 6$) after 4 week, while that of those in the amiodarone group was 336 ± 20 g ($n = 6$) after 1 week and 415 ± 28 g ($n = 6$) after 4 weeks of treatments. Thus, the increase in body weight between 1 and 4 weeks was significantly disturbed by the amiodarone treatments.

Isolation of cells

Guinea pigs were anesthetized by intraperitoneal injection of pentobarbitone. The chest was opened under artificial ventilation. The aorta was cannulated in situ, and the heart was removed. After washing out the blood with Tyrode solution, the heart was mounted in a Langendorff perfusion system and the perfusate was changed to a nominally Ca^{2+} -free Tyrode solution and then to the one containing 0.01% w/v collagenase (Wako, Osaka) and 0.002% w/v alkaline protease (Nagase, Tokyo). After digestion for about 20 min, the heart was perfused with a high K^+ , low Cl^- solution [modified KB solution (28)]. Cardiac ventricular tissue was cut into small pieces in modified KB solution and gently shaken to isolate ventricular cells. The cell suspension was stored in a refrigerator (4°C). During perfusion, the temperature of the bath solution was maintained at $36 \pm 0.5^\circ\text{C}$ with a water jacket. Tyrode solution contained 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 0.33 mM NaH_2PO_4 , 5.5 mM glucose and 5 mM HEPES-NaOH (pH 7.4). Modified KB solution contained 70 mM KOH, 50 mM *L*-glutamic acid, 40 mM KCl, 20 mM taurine, 20 mM KH_2PO_4 , 3 mM MgCl_2 , 10 mM glucose, 0.2 mM EGTA and 10 mM HEPES (pH 7.2 with KOH).

Measurement of thyroid stimulating hormone (TSH) and T_4

To assess thyroid gland function, 3–5 ml blood samples were taken from the left ventricle before extracting the heart. TSH and thyroxine (T_4) were measured at BML, Inc. (Koriyama).

Patch-clamp recording

Membrane currents and action potentials were recorded by the whole-cell voltage clamp and the current-clamp method, respectively (29). Single ventricular cells were placed in a recording chamber on the stage of an inverted microscope (Nikon, Tokyo) and superfused with Tyrode's solution at a rate of 5 ml/min. The temperature of the external solution was maintained at $36 \pm 0.5^\circ\text{C}$. Patch pipettes were forged from glass capillaries having diameters of

1.5 mm with a microelectrode puller (model pp-83; Narishige, Tokyo). The pipette resistance was 2–3 M Ω when filled with the pipette solution. Patch-clamp amplifiers for the whole cell voltage clamp were TM-1000 (Act ME, Tokyo) and EPC-7 (List, Darmstadt, Germany), and the latter was also used for recording action potentials with the current clamp method. Current signals were filtered at 2.5-kHz bandwidth. The signals were stored on-line and analyzed with a computer (PC-9801RX; NEC, Tokyo).

The action potential was recorded in the current clamp mode from single cardiac ventricular myocytes stimulated at 0.1 Hz with Tyrode's extracellular solution and a pipette solution containing 120 mM KOH, 20 mM KCl, 50 mM DL-aspartic acid, 5 mM MgATP, 3 mM MgCl_2 , 20 mM BAPTA and 20 mM HEPES (pH 7.2 with KOH). The current-voltage (*I*-*V*) relationships were obtained by ramp pulses stimulated 0.1 Hz from a holding potential of -60 mV, initially depolarized to 60 mV, then hyperpolarized to -110 mV, and depolarized back to -60 mV at a speed of 640 mVs $^{-1}$. The descending limb current (from 60 to -110 mV) was used to plot I_{NCX} (25, 26). For recording I_{NCX} , the pipette solution contained 20 mM NaCl, 20 mM BAPTA, 13 mM CaCl_2 (free Ca^{2+} concentration, 433 nM), 120 mM CsCl, 8 mM MgCl_2 , 50 mM Aspartic acid, 5 mM Na_2ATP and 10 mM HEPES (pH 7.2 with CsOH). The extracellular solution contained 140 mM NaCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 0.02 mM ouabain, 0.01 mM nifedipine, 0.005 mM ryanodine and 5 mM HEPES-CsOH (pH 7.2).

RT-PCR

Total RNA was extracted from ventricular myocytes using the acid-guanidinium thiocyanate phenol chloroform method (30). The first strand cDNA was prepared from 1 μg of total RNA with random primers using moloney murine leukemia virus reverse transcriptase in a final volume of 20 μl . The cDNA was then diluted with 80 μl of sterile water and used as the template in PCR. DNA amplification was carried out in 10 μl of solution containing 10 mM Tris-HCL (pH 9.0), 50 mM KCl, 1.5 mM MgCl_2 , 125 μM deoxynucleotide triphosphates, the cDNA template (1 μl), 1 μM primer mix and 25 units/ml Taq polymerase (Pharmacia, Tokyo). The thermocycle consisted of an initial 2-min incubation at 94°C , followed by 20–26 cycles of 30 s at 94°C , 30 s at 56°C , and 2 min at 72°C . The incubation at 72°C in the final cycle was extended to 10 min. Sequences of PCR primers for NCX cDNA were 5'-CTTCGTCCACCTACAGAAT-3' (sense strand, corresponding to bases 2384–2403) and 5'-TGGTAGATGGCAGCAATGGA-3' (antisense strand, corresponding to bases 2892–2673). As a control for RT-PCR, 500 bp β -actin cDNA fragments were amplified with sense primer (5'-GAAGCAGGGTGGATGTCTTA-3') and antisense

primer (5'-ACCATCTACCGTCGTTACCT-3'). PCR products were separated by electrophoresis in 1.5% agarose gels. After staining with ethidium bromide, PCR products were quantitated by NIH Image 1.62 software.

Drugs

Amiodarone, ouabain, nifedipine, and ryanodine were purchased from Sigma Chemical Co., St. Louis, MO, USA. KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate) was a kind gift from Kanebo Co., Ltd. (Osaka). Amiodarone was dissolved in physiological saline (15, 31). Nifedipine and KB-R7943 were first dissolved in dimethylsulfoxide (DMSO) and added to extracellular solutions so that the final concentration of DMSO was $\leq 0.1\%$, which did not affect I_{NCX} . All the chemicals were the highest grade available.

Data analyses

All values are presented as means $\pm$ S.E.M. (number of experiments). Student's *t*-test and analysis of variance were used for statistical analyses. *P* values less than 0.05 were considered significant.

RESULTS

Chronic effect of amiodarone on action potentials

It has been shown that chronic administration of amiodarone prolongs the APD in guinea pig, rabbit and canine cardiac myocytes (9, 17, 32). Therefore, APD prolongation can be used as an indicator of amiodarone exerting electrophysiological effects. Using the current-clamp method, we first examined the action potentials in guinea pig ventricular cells treated with saline or amiodarone for 1 and 4 weeks. Figure 1 shows typical action potentials recorded from cells under these conditions. APDs were measured at 90% repolarization (APD_{90}) and the values are summarized in the right panels of Fig. 1, A and B. APDs of amiodarone-treated cells were significantly longer than those of saline cells and they were longer in cells treated for 4 weeks than in cells treated for 1 week. The average resting potentials were about -72 mV and were not significantly different in amiodarone-treated and non-treated cells or in those treated for 1 and 4 weeks (Fig. 1).

Fig. 1. Typical action potentials recorded in ventricular cells from saline (left) and amiodarone (AM) treated (middle) guinea pigs. A: 1 week of treatment with amiodarone. B: 4 weeks of treatment with amiodarone. Summarized data (right) of action potential duration measured at 90% repolarization (APD_{90}). The values are expressed as means $\pm$ S.E.M. (18–29 cells from 6 guinea pigs). **P* < 0.05, based on unpaired *t*-test.

Chronic effect of amiodarone on I_{NCX}

I_{NCX} was induced with 1 mM Ca^{2+} and 140 mM Na^+ in the external solution and 20 mM Na^+ and 433 nM free Ca^{2+} in the pipette solution with a holding potential of -60 mV. KB-R7943 at 100 μ M was added to the external solution to inhibit I_{NCX} completely (29, 33). Figure 2 shows typical pairs of I-V relationships in saline and cells with KB-R7943 from guinea pigs treated with saline for 1 week (Fig. 2A, left), with amiodarone for 1 week (Fig. 2A, middle), with saline for 4 weeks (Fig. 2B, left) and with amiodarone for 4 weeks (Fig. 2B, middle). Net I_{NCX} densities measured at 50 mV are summarized in the right panels of Fig. 1, A and B. I_{NCX} density was not significantly different in the saline and amiodarone groups for both 1- and 4-week treatments, indicating that chronic treatment with amiodarone does not affect I_{NCX} .

RT-PCR analysis

Although there was no differences in I_{NCX} between the saline and amiodarone-treated groups, there might be a change in mRNA level of NCX. Therefore, we examined whether the level of NCX1 mRNA was affected by amiodarone-treatment using RT-PCR with oligonucleotide primers specific for the guinea pig cardiac NCX1 (34). Figure 3 (upper panels) shows a single 309-bp band corresponding to NCX1 mRNA and a 500-bp band corresponding to

β -actin. RT-PCR products of NCX1 and β -actin were the same in the saline and amiodarone groups treated for 1 and 4 weeks. Figure 3 (lower panels) show the average ratios of NCX1 and β -actin RT-PCR products in 1- and 4-week-treated guinea pigs. These results indicate that chronic administration of amiodarone does not affect NCX1 mRNA levels in guinea pig ventricular myocytes.

Chronic effects of amiodarone on thyroid function

Since it has been reported that chronic treatment with amiodarone could affect thyroid function, we measured T_4 and TSH in the blood of the guinea pigs. As shown in Fig. 4, the level of T_4 was the same in the saline and 4 weeks amiodarone treated groups. TSH was too low to detect in both groups.

DISCUSSION

In this study, chronic treatment of amiodarone for 1 and 4 weeks prolonged APD_{90} in guinea pig ventricular cells, indicating that amiodarone exerted its electrophysiological effects on the cardiac myocyte. However, I_{NCX} and mRNA of NCX were not changed. The prolongation of APD has been attributed to suppression of various K^+ channels, including I_{Kr} , I_{Ks} , and I_{to} (15, 17, 32). $Kv1.5$ mRNA was decreased in cardiac myocytes (17, 32). Therefore, the

Fig. 2. Comparison of I_{NCX} between 1 and 4 weeks of amiodarone treatment using ventricular cells. A: I-V curves after 1 week of amiodarone treatment with control cells (C) and in the presence 100 μ M KB-R7943 (K) which inhibited I_{NCX} completely. The right panel indicates I_{NCX} densities measured by subtraction at +50 mV of C and K. B: I-V curves recorded with cells from saline (left) and animals treated for 4 weeks with amiodarone (middle). I_{NCX} densities (right) between C and K at +50 mV are summarized. The values are expressed as means $\pm$ S.E.M. (17–32 cells from 6 guinea pigs).

Fig. 3. Effects of 1 and 4 weeks of amiodarone treatments on NCX mRNA levels in ventricular cells. Upper panels: PCR products of NCX (309 bp) and β -actin (500 bp) are shown as indicated. Lower panels: Quantitative comparison of the ratios of RT-PCR products of NCX and β -actin primers in guinea pig hearts after 1 (left) and 4 (right) weeks of amiodarone treatments. The values are expressed as means $\pm$ S.E.M. from 3 or 4 guinea pig hearts.

Fig. 4. Comparison of thyroid hormone (T_4) between saline and 4-week amiodarone-treated groups. The values are expressed as means $\pm$ S.E.M. from 4 guinea pig hearts.

synthesis of K^+ channel mRNAs may be impaired by amiodarone. This indicates that the mechanism regulating NCX mRNA expression is different from those of K^+ channels.

One of the well-known side effects of amiodarone is alteration of thyroid function. Amiodarone is a benzofuran derivative structurally similar to the thyroid hormones. Each amiodarone molecule contains two iodines, and 10% of the iodine becomes free iodine (20). Chronic treatment with amiodarone has been shown to decrease plasma levels of 3,5,3'-triiodothyronine (T_3) and to increase serum T_4 and 3,3,5'-triiodothyronine reverse T (rT_3) (35). For the mechanism of the decreasing effect of K^+ current by amiodarone, it has been suggested that long-term treatment with amiodarone may antagonize T_3 at a cellular or subcellular level and thereby counteract its hormonal effects on K^+ channels (35). In this study, chronic administration of amiodarone did not change serum T_4 . This does not necessarily mean that thyroid function was not altered in our preparation, because we did not measure serum T_3 . Since APD was prolonged, amiodarone likely affected thyroid function, possibly by interacting with T_3 receptors in the ventricular cells, as was suggested by Guo et al. (35). However, this mechanism does not seem to apply NCX.

Hojo et al. (36) showed that thyroid hormone stimulated NCX in cultured neonatal rat cardiocytes. In contrast, Boerth and Artman (37) showed that NCX mRNA levels were decreased by hyperthyroidism and increased by hypothyroidism in adult and neonate rabbit hearts. Cernohorský et al. (38) also showed that hyperthyroidism reduced NCX, while hypothyroidism increased NCX mRNA and protein in developing rat heart. The level of cardiac NCX protein was elevated in rat by chronic treatment with amiodarone (40 mM/kg body weight intraperitoneally for 6 weeks) (31). Therefore, we expected that amiodarone would modulate thyroid function and affect the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. However, in our hands, the serum level of T_4 did not change from either 1 or 4 weeks of treatment with amiodarone, and I_{NCX} and the level of NCX mRNA were not affected. One possibility was that the level of alteration of thyroid function was undetectable by T_4 and yet it was sufficient for K^+ channel modulation but not enough for NCX mRNA. Further study is required for the unanswered relation among amiodarone, NCX and the thyroid function.

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